Molecular Imaging of Pancreatic Cancer in an Animal Model Using Targeted Multifunctional Nanoparticles

Lily Yang, Emory University
Hui Mao, Emory University
Zehong Cao, Emory University
Y. Andrew Wang, Department of Ocean Nanotech, LLC
Xianghong Peng, Emory University
Xiaoqian Wang, Emory University
Hari K. Sajja, Emory University
Liya Wang, Emory University
Hongwei Duan, Emory University
Chunchun Ni, Emory University

Only first 10 authors above; see publication for full author list.

Journal Title: Gastroenterology
Volume: Volume 136, Number 5
Publisher: Elsevier: 12 months | 2009-05, Pages 1514-25.e2
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1053/j.gastro.2009.01.006
Permanent URL: http://pid.emory.edu/ark:/25593/fjbm2

Final published version: http://dx.doi.org/10.1053/j.gastro.2009.01.006

Copyright information:
© 2009 by the AGA Institute
Accessed February 13, 2020 1:50 AM EST
Molecular Imaging of Pancreatic Cancer in an Animal Model Using Targeted Multifunctional Nanoparticles

Lily Yang, Hui Mao, Zehong Cao, Y. Andrew Wang, Xianghong Peng, Xiaoxia Wang, Hari K. Sajja, Liya Wang, Hongwei Duan, Charles A. Staley, William C. Wood, Xiaohu Gao, and Shuming Nie

Department of Surgery, Emory University School of Medicine, Atlanta, Georgia
Department of Radiology, Emory University School of Medicine, Atlanta, Georgia
Department of Biomedical Engineering, Emory University School of Medicine, Atlanta, Georgia
Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia
Department of Ocean Nanotech, LLC, Springdale, Arkansas

Abstract

Background & Aims—Identification of a ligand/receptor system that enables functionalized nanoparticles to efficiently target pancreatic cancer holds great promise for the development of novel approaches for the detection and treatment of pancreatic cancer. Urokinase plasminogen activator receptor (uPAR), a cellular receptor that is highly expressed in pancreatic cancer and tumor stromal cells, is an excellent surface molecule for receptor-targeted imaging of pancreatic cancer using multifunctional nanoparticles.

Methods—The uPAR-targeted dual-modality molecular imaging nanoparticle probe is designed and prepared by conjugating a near-infrared dye-labeled amino-terminal fragment of the receptor binding domain of urokinase plasminogen activator to the surface of functionalized magnetic iron oxide nanoparticles.

Results—We have shown that the systemic delivery of uPAR-targeted nanoparticles leads to their selective accumulation within tumors of orthotopically xenografted human pancreatic cancer in nude mice. The uPAR-targeted nanoparticle probe binds to and is subsequently internalized by uPAR-expressing tumor cells and tumor-associated stromal cells, which facilitates the intratumoral distribution of the nanoparticles and increases the amount and retention of the nanoparticles in a tumor mass. Imaging properties of the nanoparticles enable in vivo optical and magnetic resonance imaging of uPAR-elevated pancreatic cancer lesions.

© 2009 by the AGA Institute

Address requests for reprints to: Lily Yang, MD, PhD, Department of Surgery and Winship Cancer Institute, Emory University School of Medicine, C-4088, 1365 C Clifton Road NE, Atlanta, Georgia 30322. lyang02@emory.edu; fax: (404) 778-5530; or Shuming Nie, PhD, Department of Biomedical Engineering, Emory University School of Medicine, 101 Woodruff Circle, Suite 2007B, Atlanta, Georgia 30322. snie@emory.edu; fax: (404) 727-3567.

X.G.'s current affiliation is: Department of Bioengineering, University of Washington, Seattle, Washington. L.Y. and H.M. contributed equally to this manuscript.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi:10.1053/j.gastro.2009.01.006.

Conflicts of interest

Y.A.W. discloses that he is the president and principal scientist of Ocean Nanotech LLC. The remaining authors disclose no conflicts.
Conclusions—Targeting uPAR using biodegradable multifunctional nanoparticles allows for the selective delivery of the nanoparticles into primary and metastatic pancreatic cancer lesions. This novel receptor-targeted nanoparticle is a potential molecular imaging agent for the detection of pancreatic cancer.

Pancreatic cancer is one of the leading causes of cancer-related death, with a 5-year survival rate of less than 5%.\textsuperscript{1} About 80% of patients present with either locally advanced or metastatic disease, which is not resectable by surgery.\textsuperscript{1} Novel approaches for early detection and effective treatment will make the most significant impact on clinical management of this disease. Recent advances in molecular imaging using biomarker-targeted contrast agents have shown enhanced specificity and sensitivity in in vivo tumor imaging.\textsuperscript{2-5} The development of multifunctional nanoparticles with capabilities of tumor targeting, imaging, and delivery of therapeutic agents may further advance our abilities in the detection and treatment of pancreatic cancer.\textsuperscript{6,7} However, a major challenge in application of tumor-targeted nanoparticles is insufficient intratumoral distribution of the nanoparticles due to disordered tumor vasculatures, low efficiency of the nanoparticles in crossing the endothelium, and extensive tumor stroma that limits nanoparticles interacting with tumor cells. One of the major pathological features of the pancreatic cancer is the presence of desmoplasia, which is the host stromal response to an invasive carcinoma.\textsuperscript{8} To increase the targeting ability of nanoparticles in pancreatic cancer, it is better to use a cellular surface target that is up-regulated in both pancreatic cancer and tumor stromal cells but is absent or poorly expressed in normal tissues.

Urokinase plasminogen activator (uPA) is a serine protease that regulates matrix degradation, cell invasion, and angiogenesis through interaction with its cellular receptor (uPAR).\textsuperscript{9,10} uPAR is highly expressed in more than 86% of pancreatic cancer tissues, but its expression is not found in pancreatic tissues obtained from healthy subjects or patients with chronic pancreatitis.\textsuperscript{9,11,12} A recent study showed that of 27 genes that are up-regulated in pancreatic cancer tissues, the level of uPAR has the highest diagnostic accuracy for the discrimination of pancreatic ductal carcinoma from chronic pancreatitis.\textsuperscript{12} The binding of uPA to uPAR occurs with a high affinity through the amino-terminal fragment (ATF, residues 1–135 amino acids) with a $K_d$ less than 1 nmol/L.\textsuperscript{13} Studies showed that the ATF peptide can compete with uPA for the binding of uPAR at the surface of both tumor and endothelial cells, resulting in inhibition of tumor growth and angiogenesis.\textsuperscript{14,15} For the development of uPAR-targeted imaging, the most important characteristic might be the ability of uPAR to efficiently internalize into cells upon binding to its ligands.\textsuperscript{16} To develop uPAR-targeted nanoparticles for tumor imaging and drug delivery, we have produced a recombinant ATF peptide that can be conjugated to a magnetic iron oxide (IO) nanoparticle (ATF-IO). IO nanoparticles are strong contrast agents for magnetic resonance imaging (MRI), which offer superb resolution of tissue morphology and anatomical details for imaging of pancreatic tissues.\textsuperscript{4,17} With its low toxicity in humans, a high capacity for carrying therapeutic agents, and strong contrast-enhancing effect, IO nanoparticles are an attractive platform for engineering uPAR-targeted multifunctional nanoparticles for pancreatic cancer imaging and therapy. In this study, we found that systemic delivery of ATF-IO nanoparticles leads to their selective accumulation in tumors of an orthotopic human pancreatic cancer xenograft model and demonstrated uPAR-targeted molecular imaging of pancreatic cancer with a multifunctional IO nanoparticle probe.
Materials and Methods

Cell Lines

Human pancreatic cancer MIA PaCa-2 and breast cancer T47D cell lines were purchased from American Type Culture Collection (Rockville, MD). MS1 murine endothelial cell line was provided by Dr Jack Arbiser at Emory University. Normal human pancreatic duct epithelial cell line HPDE6 was obtained from Dr Ming-Sound Tsao at University of Toronto, Canada.

Immunostaining

Frozen sections of pancreatic normal and cancer tissues were obtained from surgically removed pancreatic tissues according to an approved institutional review board protocol at Emory University. Human pancreatic tumor xenografts were obtained from orthotopic pancreatic tumors derived from the MIA PaCa-2 cell line. Frozen tissue sections were incubated with an anti-uPAR polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and followed by Alexa Fluor 555 goat anti-rabbit immunoglobulin G (Invitrogen, Carlsbad, CA). Human or mouse endothelial cells were identified using a monoclonal anti-human CD31 or a fluorescein isothiocyanate–labeled rat anti-mouse CD31 antibody (BD Biosciences, San Diego, CA). Mouse macrophages were identified using Alexa Fluor 488–labeled rat anti-mouse CD68 antibody (AbD Serotec, Raleigh, NC).

Immunochemical staining was performed by incubating tissue sections with an anti-uPAR antibody and then with alkaline phosphatase–labeled anti-rabbit antibody followed by Alkaline Phosphatase Substrate Kit I (Vector Laboratories, Burlingame, CA). Expression of a tumor marker carcinoembryonic antigen was determined using a monoclonal anti–carcinoembryonic antigen antibody (Sigma, St Louis, MO) and horseradish peroxidase–labeled anti-mouse antibody, followed by DAB Peroxidase Substrate Kit (Vector Laboratories).

Production of Recombinant Mouse ATF Peptidess

A complementary DNA fragment encoding amino acids 1 to 135 of mouse uPA was isolated by polymerase chain reaction amplification and then cloned into pET101/D-TOPO expression vector (Invitrogen). Recombinant ATF peptides were expressed in Escherichia coli BL21 (Invitrogen) and purified from bacterial extracts under native conditions using an Ni²⁺-NTA-agarose column (Qiagen, Valencia, CA).

Cy5.5-labeled ATF peptide was produced by conjugating Cy5.5 maleimide to ATF peptides using the manufacturer’s protocol (GE Healthcare, Piscataway, NJ). Free dye molecules were separated using a Nanosep 3k OMEGA column (Pall Corp, Ann Arbor, MI).

Preparation of uPAR-Targeted ATF-IO and Cy5.5-ATF-IO or Control Cy5.5-IO Nanoparticles

Size-uniformed IO nanoparticles were prepared as described. The IO nanoparticles (10 nm core size) were coated with amphiphilic polymers. ATF peptides or Cy5.5-ATF peptides were then conjugated to the IO nanoparticles via cross-linking of carboxyl groups of the amphiphilic polymers to amino side groups of the peptides (Figure 2A). The final ATF-IO nanoparticle conjugates were purified using Nanosep 100k column. The conjugation of ATF peptides to the IO nanoparticles was determined by fluorescence spectroscopy using Cy5.5-ATF peptides. To prepare the Cy5.5-IO nanoparticles as a control, the Cy5.5-mono NHS ester was directly labeled to the amino group on the amphiphilic polymer after being modified with N-Boc-1,6-hexanediamin.
Specificity of ATF-IO Nanoparticles

Cells were incubated with nanoparticles for 3 hours at 37°C. To determine target specificity, some cells were transfected with human uPAR small interfering RNA (siRNA) or control siRNA for 24 hours (Applied Biosystems, Austin, TX) or preincubated with 20 µg/mL of recombinant ATF peptides for 1 hour before adding ATF-IO nanoparticles. The cells were examined using Prussian blue staining to determine the presence of IO nanoparticles.

For MRI experiments, $1 \times 10^7$ cells were incubated with 10 nmol/L per milliliter of nontargeted or ATF-IO nanoparticles at 37°C for 3 hours. Cells were washed and then embedded in 1% agarose in multi-well plates. The plates were then scanned in a 3T MRI scanner (Philips Healthcare, Best, The Netherlands) using $T_1$-weighted gradient echo and multi-echo $T_2$ weighted fast-spin echo imaging sequences. $T_2$ values of each sample were calculated from obtained multi-echo ($T_{E_i}$, $i = 25$) $T_2$-weighted MR images by fitting the decay curve on a pixel-by-pixel basis using the nonlinear mono-exponential algorithm of $M_i = M_0 \cdot \exp (-T_{E_i}/T_2)$.

Orthotopic Human Pancreatic Cancer Xenograft Model

The pancreatic tumor model was established by injecting $5 \times 10^6$ of MIA PaCa-2 cells into the pancreas of the 6- to 8-week-old female nude mice (Taconic, Hudson, NY) using a surgical procedure approved by the Institute of Animal Use Committee of Emory University. Orthotopically xenografted pancreatic tumors reached 5 mm in diameter and were ready for experiments in about 3 to 4 weeks.

In Vivo MRI

Tumor-bearing mice were scanned using a 3T MRI scanner with a 5-cm volumetric coil. The imaging sequences include $T_1$- and $T_2$-weighted spin echo or gradient echo methods. A multi-echo ($T_{E_i}$, $i = 12$) $T_2$-weighted fast-spin echo sequence was used to obtain $T_2$ relaxometry of the mouse. After the mice were injected with 200 to 300 pmol of various nanoparticles through the tail vein, they were imaged at different time points to follow the changes of MRI signals. Images from precontrast and postcontrast administration were compared to evaluate the efficacy of contrast enhancement. The region of interest method was used to evaluate and quantify the contrast agent–induced image contrast and $T_2$ value changes in the tumor.

Near-Infrared Fluorescence Optical Imaging

The mice were placed on an alfalfa-free rodent diet (Harlan Teklad, Madison, WI) to reduce background fluorescence. Optical imaging was performed using the Kodak In Vivo Imaging System FX (Carestream Health, Inc, New Haven, CT). All images were taken using a 625-nm excitation and 700-nm emission filter set with a 40-second exposure time and a $\gamma$ value of 0.2. For each near-infrared fluorescence (NIR) image, a corresponding x-ray image was taken to provide anatomic location of the tumor.

Histologic Analysis

The mice were sacrificed after the imaging study was completed. Tumor and normal tissues were collected, and ex vivo optical imaging was performed on the tissues. The frozen tissue sections were examined by Prussian blue staining and immunolabeling.
Results

uPAR Is Up-Regulated in Human Pancreatic Cancer Tissues and Tumor Xenografts

To validate uPAR as a molecular target, we examined its expression in human pancreatic cancer tissues and found a high level of expression in pancreatic cancer but not in normal pancreatic tissues. Human tumor endothelial cells were also positive for uPAR (Figure 1A). In pancreatic tumor xenografts obtained from nude mice, high levels of uPAR expression were detected in the cancer cells and tumor stromal cells such as endothelial cells and macrophages that originated from mice (Figure 1B). However, uPAR was not detected in the normal pancreatic tissues (Figure 1C). uPAR was also undetectable in the majority of normal tissues and organs, except for a small percentage of cells in the spleen. Lung and kidney have a low level of uPAR (Figure 1C).

uPAR-Targeted Nanoparticles Bind to and Are Internalized by Tumor and Endothelial Cells

Mouse ATF peptide conjugated-IO nanoparticles were incubated with cancer and endothelial cell lines in vitro. Prussian blue staining revealed iron-positive cells in uPAR-expressing human pancreatic cancer and mouse endothelial cell lines after incubating with ATF-IO nanoparticles but not with nontargeted IO nanoparticles (Figure 2B). Mouse endothelial cells had a higher level of iron staining compared with human pancreatic cancer cells. The T47D cell line does not express a detectable level of uPAR and was also negative (Figure 2B). The binding and internalization of the targeted nanoparticles are further confirmed by significant reduction of Prussian blue–stained MIA PaCa-2 cells after transfecting the cells with uPAR siRNA or blocking with excessive amounts of free ATF peptides (Figure 2C).

MRI scan of the cells after incubating with ATF-IO nanoparticles further demonstrated target specificity, because MRI signal reduction was detected in uPAR expressing cells. IO nanoparticles typically yield MRI contrast with signal reduction due to the T₂ relaxation time shortening effect. The T₂ relaxometry measurement revealed the largest T₂ reduction and signal decrease in MS1 (Figure 2D), indicating that higher amounts of IO nanoparticles are internalized in these cells. Our results also showed that IO nanoparticles conjugated with mouse ATF were able to bind to human cancer cells through cross-reactivity. There was no MRI contrast change following incubation of ATF-IO nanoparticles with a normal human pancreatic ductal cell line HPDE (Figure 2D).

In Vivo MRI Shows That ATF-IO Nanoparticles Target Tumors in an Orthotopic Human Pancreatic Cancer Xenograft Model

When comparing the T₂-weighted MR images of the tumor-bearing mice before and after administration of IO nanoparticle, marked signal decreases were detected in the tumor area 5 to 20 hours following injection of the targeted nanoparticles, suggesting selective accumulation of the nanoparticles in the pancreatic cancers (Figure 3A). In contrast, there was no apparent MRI signal change in the tumors of mice receiving nontargeted IO nanoparticles (Figure 3B). In the other control experiment, we did not see an MRI contrast change in the pancreas of normal mice after administration of ATF-IO nanoparticles (Figure 3C). Notably, signal decreases were also seen in the liver and spleen (Figure 3A–C).

Histologic analysis of tumor and normal tissues revealed significant iron-positive cells in pancreatic tumors but not in surrounding normal pancreatic tissues in mice that received ATF-IO nanoparticles for 96 hours (Figure 3D). Iron-positive cells were also detected in the liver and spleen of the mice that received ATF-IO nanoparticles, but their levels were significantly lower compared with those in the mice receiving nontargeted IO nanoparticles (Figure 3D). The normal pancreas, stomach, intestines, brain, and heart tissues were
consistently negative for iron staining, and only a few scattered iron-positive cells were
detected in the kidney and lung in the animals receiving ATF-IO nanoparticles (Figure 3E).
We did not find apparent histologic abnormality in these normal tissue sections.

**Optical Imaging Demonstrates uPAR-Targeting Specificity, Distribution, and Time-
Dependent Clearance of ATF Nanoparticles in Tumor-Bearing Mice**

Because optical imaging is a simple and sensitive imaging modality that allows for real-time
visualization of tissue distribution of nanoprobes in mice, we examined tumor-bearing mice
injected with Cy5.5-ATF peptides or Cy5.5-ATF-IO or Cy5.5-IO nanoparticles at different
time points. We found that although Cy5.5-ATF peptides alone could emit an NIR signal in
the tumor 24 to 72 hours after injection, the signal intensity was 3- and 4-fold weaker at 48
and 72 hours compared with that in the mice receiving Cy5.5-ATF-IO nanoparticles (Figure
4A, B, and E). A strong signal was detected in the bladder 2 hours after the peptide
injection, suggesting that a large amount of Cy5.5-ATF peptides was secreted through the
kidney (Figure 4A). In mice that received Cy5.5-ATF-IO nanoparticles, the NIR signal was
found in pancreatic cancer 24 hours after injection. The intensity of the signal in the tumor
continued to increase 48 to 96 hours after injection (Figure 4B and E). Ex vivo NIR imaging
of the tumor and normal tissues confirmed the presence of a strong NIR signal in the tumor
but not in most normal tissues except the kidney and bladder (Figure 4B). In a mouse that
received a nontargeted Cy5.5-IO nanoparticle injection, a strong NIR signal was detected in
the liver 5 hours after injection and lasted for more than 48 hours (Figure 4C). NIR signals
from the kidneys were detectable when imaging of the mice was performed from the back
(Figure 4D). Taken together, our results suggest that Cy5.5-ATF-IO nanoparticles
selectively accumulate in pancreatic cancers after systemic delivery and that nanoparticles
allow for prolonging the blood and tumor retention time of the imaging probe and thus
enhance the tumor targeting effect and signal of NIR imaging.

**Dual-Modality Imaging Using Cy5.5-ATF-IO Nanoparticles Detects Primary and Metastatic
Pancreatic Tumors**

Given the advantages of sensitivity of optical imaging and high resolution of MRI, we
investigated the target specificity of Cy5.5-ATF-IO nanoparticles in mice bearing primary
and intraperitoneal metastatic lesions. We found that NIR optical imaging clearly delineated
4 tumor lesions in the peritoneal cavity with volumes ranging from 0.5 to 100 mm³ (Figure
5A and C). MRI and T2 relaxometry measurement performed on the same mouse revealed
MR signal decrease in 2 primary tumor nodules (Figure 5B), even though the sensitivity of
MRI is lower than NIR.

**ATF-IO Nanoparticles Target Tumor Cells and Tumor Stromal Cells In Vivo**

We examined the cell types in the tumor mass that bind to ATF-IO nanoparticles and found
that iron-positive cells are colocalized with uPAR-positive tumor cells. Interestingly, iron-
positive cells were abundant in both the tumor and stroma immediately adjacent to the tumor
cells, but not in surrounding normal pancreatic tissue (Figure 6A). Some CD31-positive
tumor vessels were also positive for iron staining (Figure 6B). Iron-positive cells were
detected in both CD68-positive macrophage and CD68-negative cells, suggesting that MRI
contrast in the tumor may be caused by the uptake of ATF-IO nanoparticles in both tumor
cells and tumor-associated macrophages (Figure 6C). Although we detected CD68-positive
macrophages in the lung tissue sections, these cells did not have positive iron staining
(Figure 6C). Additionally, almost all iron-positive cells in the liver were C68-positive
macrophages or Kupffer cells.
Sensitivity of NIR Optical and MR Imaging in Pancreatic Cancer Xenograft Models

Our results further showed that pancreatic tumors as small as 1 mm³ can be detected by a 3T MRI scanner at the clinical field strength (Figure 7A), while an intraperitoneal pancreatic cancer lesion with a size of 0.5 mm³ was also detected by NIR optical imaging (Figure 5C) after systemic delivery of ATF-IO or Cy5.5-ATF-IO nanoparticles. To precisely determine the number of Cy5.5-ATF-IO nanoparticle-labeled cells that produce sufficient signal for NIR optical or detectable contrast for MR imaging, MIA PaCa-2 cells were labeled with Cy5.5-ATF-IO nanoparticles in culture and different amounts of labeled cells were then injected into the pancreas of nude mice. We found that NIR optical imaging detected tumor cell implants with as little as $1 \times 10^4$ tumor cells while MRI detected tumor cell grafts containing $1 \times 10^5$ labeled cells (Figure 7B and C).

Discussion

Biomarker-targeted imaging and treatment hold great promise for improving the survival of patients with cancer. In the case of pancreatic cancer, there is no reliable imaging approach for diagnosis. Cell surface biomarkers that allow for the development of targeted imaging contrast agents and/or therapeutics for pancreatic cancer have yet to be identified.

In this study, we selected the ATF peptide as a targeting ligand with the rationale that the high binding affinity of ATF with uPAR is able to block the interaction of uPAR with its natural ligand uPA and that delivery of ATF peptides inhibits tumor growth and angiogenesis in several animal tumor models.14,20 The ability of targeted nanoparticles to be efficiently taken up by cancer cells is important for increasing their tumor-specific accumulation for tumor imaging and for delivering therapeutic agents. The results of our study showed that ATF-conjugated nanoparticles specifically bind to uPAR and are internalized by the cells. In vivo systemic delivery of ATF-IO nanoparticles leads to their selective accumulation in orthotopic pancreatic tumors, as shown by both optical and MR imaging. In this study, we used mouse ATF to generate uPAR-targeted IO nanoparticles, which is appropriate for the investigation of target specificity and tissue distribution of the nanoparticle in mouse. Although we observed that mouse ATF peptides cross-react with human tumor cells, we have produced human ATF peptide for future clinical application because it has a higher binding affinity for human uPAR and is not immunogenic in humans.

An important aspect for increasing the sensitivity of in vivo tumor imaging as well as the efficiency of targeted therapy is to deliver large amounts of the nanoparticles into a tumor mass. Currently used target molecules, such as Her-2/neu and MUC-1, are expressed in subpopulations of tumor cells in tumor tissues.5,21 In contrast, uPAR is highly expressed in tumor and stromal cells, which increase the numbers of target cells in a tumor mass and facilitates the active retention of the nanoparticles.22,23 A recent study showed that the level of uPAR expression in both pancreatic cancer and endothelial cells is further up-regulated by tumor hypoxia, which is a common phenomenon in pancreatic cancer tissues.24 In the majority of normal tissues, uPAR is undetectable except for a low level in macrophages, granulocytes, the uterus, thymus, kidney, and spleen.25

Nanoparticles with sizes between 10 and 100 nm have a prolonged circulation time and are small enough to pass through leaky vasculatures inside tumors, making the tumor mass accessible for nanoparticles by a passive targeting mechanism26 (Figure 8). Moreover, the presence of a high level of uPAR in tumor endothelial cells may facilitate their interaction with the targeted nanoparticles, which is the first step toward the retention of the nanoparticles in the tumor. In normal tissues, an intact endothelial layer and a very low level of uPAR in endothelial cells prevent ATF-IO nanoparticles entering into the tissues. The association of uPAR with caveolae, a transcytosis machinery, may further enable
nanoparticles to cross the endothelium and rapidly enter the perivascular tumor areas\textsuperscript{27,28} (Figure 8). Targeting uPAR-expressing tumor stromal cells is important for efficient delivery of the nanoparticles into pancreatic cancers because active tumor stroma make up the majority of pancreatic cancer tissues. Our results of in vivo imaging of tumor-bearing mice after intravenous delivery of ATF-IO nanoparticles and histologic analysis of the tumor and normal tissues provide strong evidence supporting the previously proposed targeting mechanism.

The results of this study not only provide mechanistic insights into targeting uPAR for cancer imaging but also demonstrate an important approach for developing receptor-targeted multifunctional nanoparticles. The results of our time-dependent NIR imaging study suggest a clear advantage of a nanoparticle platform in tumor targeting by conjugating Cy5.5-ATF peptides to nanoparticles over Cy5.5-ATF peptides alone. Our in vivo tumor imaging results showed strong NIR signals in the pancreatic tumor areas, but not in the liver, spleen, and many other normal organs of the tumor-bearing mice that received Cy5.5-ATF-IO nanoparticles. One of the most challenging tasks in clinical diagnosis of pancreatic cancer is to distinguish between lesions of the tumor and chronic pancreatitis. Our results showed that uPAR is not up-regulated in chronic pancreatitis tissues and systemic delivery of the targeted nanoparticles does not produce optical and MR imaging signals or contrast in the pancreas of the mice with chronic pancreatitis (Supplementary Results; see supplemental material online at www.gastrojournal.org). Additionally, this biodegradable nanoparticle is a sensitive imaging probe that is able to detect tumors as small as 0.5 to 1 mm\textsuperscript{3} in animal tumor models. Therefore, it has potential for future clinical applications, such as preoperative detection and localization of pancreatic cancer lesions by MRI, intraoperative delineation of tumor margins and peritoneal metastases by optical imaging, and targeted delivery of therapeutic agents while monitoring the response to therapy using MRI in patients with pancreatic cancer.

\section*{Supplementary Material}
Refer to Web version on PubMed Central for supplementary material.

\section*{Acknowledgments}
The authors thank Drs Jack Arbiser and Ming-Sound Tsao for MS1 and HPDE6 cell lines, Dr Robert Long for assisting in magnetic resonance imaging, Dr Young-seok Cho for helping with the chronic pancreatitis model, and Dr Anthea Hammond for editing the manuscript.

\section*{Funding}
Supported by the Emory-Georgia Tech Nanotechnology Center for Personalized and Predictive Oncology of the National Institutes of Health Center of Cancer Nanotechnology Excellence (CCNE, U54 CA119338-01) and by seed grants from the Golfers Against Cancer Foundation, The Friends For An Early Breast Cancer Test, and EmTech Bio, Inc.

\section*{Abbreviations used in this paper}
\begin{itemize}
  \item \textbf{ATF} \hspace{1cm} \textit{amino-terminal fragment}
  \item \textbf{IO} \hspace{1cm} \textit{magnetic iron oxide nanoparticles}
  \item \textbf{MRI} \hspace{1cm} \textit{magnetic resonance imaging}
  \item \textbf{NIR} \hspace{1cm} \textit{near-infrared fluorescence}
  \item \textbf{siRNA} \hspace{1cm} \textit{small interfering RNA}
\end{itemize}
uPA  urokinase plasminogen activator
uPAR  urokinase plasminogen activator receptor

References


Figure 1.
Examination of uPAR expression by immunofluorescence labeling. (A) uPAR is detected in human pancreatic cancer tissues but not in normal pancreas. PC1 and PC3 are cancer tissue samples from 2 patients. Normal pancreatic tissue of the PC3 patient was used as a control. Lower panel images were taken from the same field to show uPAR-positive tumor vessels (arrows). (B) High levels of uPAR were found in orthotopically xenografted human pancreatic cancer cells, CD31-positive endothelial cells, and CD-68 positive tumor macrophages (orange yellow). (C) Double labeling of normal tissue sections of the mice with uPAR and CD31 or CD68 antibody. uPAR was not detected in the normal pancreas, liver, or heart. Weakly uPAR-positive cells were found in a subpopulation of the spleen and
scattered cells in the lung and kidney. Endothelial cells lining blood vessels and macrophages in normal tissues did not have a detectable level of uPAR expression. uPAR (red), CD68 (macrophage, green), CD31 (endothelial cells, green), Hoechst 33342 (counterstaining, blue).
Figure 2.
Production and characterization of uPAR-targeted nanoparticles. (A) Schematic illustration of conjugating ATF or Cy5.5-ATF peptides to amphiphilic polymer-coated IO nanoparticles. (B) Specific binding and internalization of ATF-IO nanoparticles. Prussian blue staining was found in the cytoplasm of MIA PaCa-2 and MS1 mouse endothelial but not T47D cells. Immunohistochemical staining using an anti-uPAR antibody detects uPAR-expressing cells (red). Blue background staining: hematoxylin. (C) Demonstration of target specificity using uPAR siRNA-transfected MIA PaCa-2 cells or by preincubating the cells with free ATF peptides. (D) MRI T2 mapping. MS1 cells showed the strongest decrease in T2 value (red). MIA PaCa-2 cells showed an intermediate level of T2 signal decrease.
(yellow), while normal human pancreatic ductal epithelial cell line HPDE and uPAR-negative T47D cells did not show an MRI contrast change.
Figure 3.
Examination of target specificity of ATF-IO nanoparticles in vivo using MRI. Mice bearing orthotopic pancreatic cancer received 200 to 300 pmol of ATF-IO or nontargeted IO nanoparticles. A control normal mouse also received the same amount of ATF-IO nanoparticles. (A) MR images of a tumor-bearing mouse before (Pre) and after injection of ATF-IO nanoparticles. Arrows indicate the location of the tumor in MR images (red) or in collected pancreatic tissue (blue). The tumor lesion is indicated as a pink dotted circle. The tumor is shown as bright contrast before the nanoparticle injection. Five to 20 hours (hrs) following the nanoparticle administration, marked MRI contrast change (darker) was detected in the tumor area. L, left; R, right. MRI contrast decrease was also detected in the
liver (yellow arrow) and spleen. Representative MR images from one of the 6 mice are shown. (B) MR images of a tumor-bearing mouse that received non-targeted IO. An MRI contrast change was only detected in the liver and spleen. There is no apparent contrast change in the tumor (arrow). (C) Delivery of ATF-IO nanoparticles in a normal mouse only induced MRI contrast change in the liver and spleen but not in the location of normal pancreas (green dotted circle). (D) Prussian blue staining showed iron-positive cells in the tumor obtained from a mouse that received ATF-IO nanoparticle (red arrow) but not in adjacent normal pancreatic tissues (green arrow) or in the tumor of the mouse injected with nontargeted IO nanoparticles. The presence of pancreatic tumor cells was confirmed by immunostaining using an anti–carcinoembryonic antigen antibody (brown arrow). (E) Examination of biodistribution of ATF-IO nanoparticles in normal tissues by Prussian blue staining.
Figure 4.
Examination of target specificity of Cy5.5-ATF-IO nanoparticles using in vivo optical imaging. Mice bearing orthotopic pancreatic cancers received Cy5.5-ATF peptides or Cy5.5-ATF-IO or non-targeted Cy5.5-IO nanoparticles. Optical imaging was performed at different time points using the same imaging parameters. The NIR signal scale was generated using the Kodak Molecular Imaging Software. Blue arrow, bladder; pink arrow, pancreatic tumor. (A) A mouse received free Cy5.5-ATF peptides. (B) NIR images of a mouse that received Cy5.5-ATF-IO nanoparticles. The result shown is a representative image from one of 4 mice. (C) Optical images of a tumor-bearing mouse that received nontargeted Cy5.5-IO nanoparticles. (D) An optical image from the back of a mouse that...
received Cy5.5-ATF-IO nanoparticles. (E) Quantitative analysis of the optical images showed that at 24 hours, a similar NIR intensity was detected in pancreatic tumors of the mice that received Cy5.5-ATF peptides or Cy5.5-ATF-IO nanoparticles. However, at 48 to 72 hours, the NIR signal intensity in the tumor area of the mice that received Cy5.5-ATF-IO nanoparticles is 3- to 4-fold higher than that of the mice that received free Cy5.5-ATF peptides. The values in the figure are the mean fluorescence intensity of 3 to 4 randomly selected tumor areas after subtracting the background fluorescence.
Figure 5.
Dual optical and MR imaging of pancreatic cancer using Cy5.5-ATF-IO nanoparticles. (A) A tumor-bearing mouse received Cy5.5-ATF-IO nanoparticles after 48 hours. Yellow arrows indicate primary (tumors #1 and #2) or metastatic (#3 and #4) pancreatic cancer lesions. A strong NIR signal was detected in the primary tumors. Tumors #3 and #4 are 1 mm$^3$ and 0.5 mm$^3$ intraperitoneal metastatic tumor lesions. A strong NIR signal was also detected in the bladder. (B) $T_2$ map obtained from the same mouse at 48 hours demonstrated a marked $T_2$ reduction in the pancreatic cancer lesion (orange-red, lower $T_2$). Blue arrow, pancreatic tumor; white arrow, spleen. (C) Ex vivo optical imaging of tumor and normal tissues collected confirmed the in vivo imaging results. Similar results were found in 3 other
mice that received Cy5.5-ATF-IO nanoparticles and had dual imaging experiments performed.
Figure 6.
Determination of cell types that bind to and internalize ATF-IO nanoparticles in pancreatic cancer tissues. (A) Prussian blue staining of a frozen tumor tissue section from a mouse that received ATF-IO nanoparticles followed by immunohistochemical staining with an anti-uPAR antibody. ATF-IO nanoparticles (blue) were found in tumor area with uPAR-positive cells (red). (B) Fluorescein isothiocyanate rat anti-mouse CD31 was used to label Prussian blue–stained tissue sections. Pink arrows indicate CD31 and iron doubly positive cells. ATF-IO nanoparticles were accumulated in a tumor border area where CD31-positive vessels were detected (red arrows). (C) Prussian blue–stained tissue sections were then labeled with an antibody to mouse CD68. Red arrows, iron-positive and CD68-negative...
cells; pink arrows, iron- and CD68-positive cells; yellow arrow, iron-negative and CD68-positive macrophages. In the liver, most CD68 and Prussian blue–positive cells were colocalized. Each panel of the images was from the same field ($B$ and $C$).
Figure 7.
Examination of sensitivity of in vivo tumor imaging. (A) uPAR-targeted MRI of an orthotopic pancreatic cancer. Delivery of ATF-IO nanoparticles by the tail vein injection allowed for detecting a small pancreatic tumor with a diameter of 1.3 mm, or a tumor volume of 1.1 mm³, by MRI (pink dotted circle). Prussian blue staining revealed the presence of IO nanoparticles in the tumor lesion with strong staining in tumor stromal areas. (B and C) MIA PaCa-2 cells were incubated with Cy5.5-ATF-IO nanoparticles for 2 hours. A total of $1 \times 10^4$ to $3 \times 10^6$ of labeled cells were then injected into the pancreas of the mice with each pancreas containing 2 cell concentrations. A total of $1 \times 10^6$ of nonlabeled cells were injected into the control mice. NIR optical imaging was performed immediately after
the surgery. MRI was conducted 24 hours later to minimize the effect of edema on MRI signal. NIR imaging detected $1 \times 10^4$ cells ($B$) and the signal enhanced as the cell number increased. MRI delineated the injection site with at least $1 \times 10^5$ of cells in the pancreas ($C$). Arrows indicate injection sites.
Figure 8.
Targeting mechanism for delivering ATF-IO nanoparticles into pancreatic cancer tissues. ATF-IO nanoparticles target to and are enriched in the tumor mainly through active targeting by interacting with uPAR-expressing tumor endothelial cells, which facilitate transportation of the nanoparticles across the blood vessel. The ability of the targeted nanoparticles to bind to and be internalized by tumor cells, active tumor macrophages, and fibroblasts enhances the delivery of nanoparticles into the tumor.